Getting Surprising Answers to Unasked Questions

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"What did you accomplish during your PhD thesis work at the University of Berne?" my late and beloved mentor Robert (Bob) P. Perry asked me when I joined his laboratory at the Fox Chase Cancer Research Institute in Philadelphia as a postdoctoral fellow in 1975. It was clearly a catch question, as he must at least have read my paper published in *Journal of Molecular Biology* before he accepted me as a lab member. Typical for somebody having been educated in Switzerland, I followed the creed of my parents and teachers that modesty is one of the noblest human virtues: "I worked on the secondary structure of ribosomal and pre-ribosomal RNAs in various vertebrate species, but my findings are not so important." I expected him to protest and to add a few praising words on my doctoral thesis projects. His answer, however, was brutal: "In that case I don't have time to listen." Several months later he confirmed my suspicion of having been tricked by his gotcha question. He wanted me to realize how stupid it would be to spend long hours on something "not so important." In the meantime, I read Arthur Bloch's *Murphy's Law: Complete*, and in the collection of truisms relevant for scientists, I found Gordon's first law: *if a research project is not worth doing at all, it is not worth doing well*. I am not sure I always followed this sagacious advice, but at least I passed it on to my pre- and postdoctoral students.

Shortly before I arrived in Philadelphia, Bob discovered methylation of messenger RNA (mRNA), which led to the identification of 5′-terminal cap structures and 6-methyl adenosines in these

Schibler's small team before moving from Lausanne to Geneva in 1984. (Left to right: Felipe Sierra, Phil Shaw, Anne-Cecile Pittet, Mauro Carneiro, and Ueli Schibler.)

transcripts. After I did some work on these, I was assigned another project: I was asked to clone cDNAs for immunoglobulin mRNAs for the purpose of studying their synthesis and processing. My lab mates Ken Marcu and Oscar Valbuena had previously succeeded in enriching these transcripts by sucrose gradient sedimentation so that they represented a sizeable fraction in the light- and heavy-chain peaks. Since the Fox Chase Cancer Institute did not have a P3 facility—then mandatory for conducting these "dangerous" cDNA cloning experiments—I was sent to the Carnegie Institution of Embryology in Baltimore, headed by Don Brown. Under the supervision of Yasumi Ohshima, a postdoc in Yoshi Suzuki's group, I managed to establish "cDNA libraries" yielding less than 50 bacterial colonies from about half a microgram of ligated recombinant DNA for each of the two RNA fractions. Luckily, I managed to identify a few cDNA clones for both light- and heavy-chain mRNAs in spite of the ridiculously low number of recombinant bacterial colonies. The *E. coli* strain χ1776, considered to be safe for recombinant DNA work, grew slower than yeast, and it took three days to see colonies on the Petri dish. In a nutshell, doing such cloning work was a nightmare back then. But this was only one of the frightening steps in conducting the project we had in mind. For the experiments aimed at following the fate of newly synthesized immunoglobulin light- and heavy-chain transcripts, I had to incubate plasmacytoma cells with tens of millicuries of ^{3}H-labeled uridine to accumulate measurable radioactivity in these RNAs. The burning hot RNA preparations were size-fractionated on cylindrical agarose gels containing the highly poisonous methylmercuric hydroxide as a denaturing agent. After cutting these gels into 60 or more serial slices, the agarose was solubilized in sodium perchlorate at 68°C, and the RNA from each slice was hybridized to a small Millipore filter containing an excess of denatured immunoglobulin recombinant DNA. Heating of the gel pieces not only liquefied the agarose but also liberated toxic methylmercury fumes. It goes without saying that I never informed my wife Monika about these hazardous undertakings. At least my pulse-labeling and pulse-chase experiments fulfilled their purpose: they revealed the processing kinetics of light- and heavy-chain immunoglobulin mRNAs from high-molecular-weight precursors. The publication of these findings in *Cell* brought me a bit closer to my dream of becoming an independent investigator.

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In 1978, I moved back to Switzerland, where I got a tenure-track group leader position at the Swiss Institute of Experimental Cancer Research (ISREC) near Lausanne. In collaboration with Peter Wellauer and Otto Hagenbüchle, also group leaders at ISREC, I initiated a project on tissue-specific gene expression. Inspired by Bill Rutter at UCSF, we chose the exocrine pancreas as our model system. In pancreatic acinar cells, about a dozen genes encoding various digestive enzymes produce more than 90% of the mRNA mass, and we expected the cloning of the corresponding cDNAs to be a piece of cake. Unfortunately, we forgot that ribonuclease A is one of the abundant pancreatic proteins, and our first efforts to extract RNA from pancreas yielded no molecules spanning more than a few hundred nucleotides. What a relief when John Chirgwin in Bill Rutter's lab established the guanidium thiocyanate/β-mercaptoethanol extraction method, now the gold standard for the isolation of intact RNA from whatever source. After one year of fruitless attempts, we were now able to purify intact polyadenylated RNA from mouse pancreas: electrophoresis of this RNA in ethidium bromide-stained methylmercuric hydroxide gels revealed about 10 fat bands, each corresponding to an mRNA specifying an abundant hydrolytic enzyme. Among those, α-amylase mRNA was the most abundant, and we thus worked hard toward obtaining α-amylase cDNA clones so that we could study its expression. Our paper on mouse α-amylase expression appeared in September of 1980, simultaneously with that of Bill Rutter and his coworkers on rat α-amylase expression. We were rookies in studying tissue-specific gene expression, and having succeeded in keeping abreast with a giant in the field lent us wings in conducting future research on the subject.

In addition to the exocrine pancreas, parotid gland and liver also express α-amylase, albeit from a different gene. Whereas pancreatic α-amylase is specified by *Amy2*, parotid gland and liver α-amylases are encoded by *Amy1*. Nonetheless, owing to the high sequence homology between *Amy1* and *Amy2* mRNAs, we could use the *Amy2* cDNA clone to detect *Amy1* transcripts in northern blot experiments with parotid and liver RNAs. Unexpectedly, liver *Amy1* transcripts consistently migrated somewhat slower than parotid gland *Amy1* transcripts on denaturing gels, even after removal of their poly(A) tails. Hence, it appeared that liver *Amy1* mRNA contained additional and/or different sequences when compared to its parotid counterpart. DNA sequencing was still not a trivial matter during the late seventies, but fortunately, Rick Young, now at the Whitehead Institute, and Otto Hagenbüchle had learned the Maxam-Gilbert method in Joan Steitz'

laboratory before they joined the ISREC. We thus cloned *Amy1* cDNA and genomic sequences, and Rick and Otto sequenced and compared the resulting recombinant DNAs. The outcome was pleasing: in parotid gland and liver, *Amy1* transcription is initiated at different promoters located 7.5 kb and 4.5 kb, respectively, upstream of the second exon. Thereby, they produced parotid and liver mRNAs with distinct non-translated exons at their 5′ termini. Today, nobody would write home about this finding, but back then, it established the first case of differential promoter usage and splicing. To our delight, this discovery was incorporated into the first editions of Benjamin Lewin's famous textbook, *Genes*. Moreover, it secured us many invitations to prestigious scientific conferences and facilitated my promotion to a tenured group leader position at ISREC in 1981. Those days, our knowledge on gene structure and transcription in mammalian cells was sketchy at best, and even studying genes encoding proteins as mundane as α-amylase culminated in publications in *Nature*, *Science*, and *Cell*.

Obviously, we would have loved to elucidate the mechanisms governing differential *Amy1* transcription in parotid gland and liver. Alas, parotid gland, with its small size and high content of digestive enzymes, was not an ideal model system for the biochemical dissection of tissue-specific transcription mechanisms. In 1984, I was hired as a professor at the University of Geneva, and moving to a new institution was soon followed by changing the experimental model system. Liver, a large, soft, and relatively homogeneous tissue with little "bad stuff," has long been known to be ideal for biochemical experiments. Since *Amy1* is weakly transcribed in hepatocytes, we decided to work on the transcription of the serum albumin gene, specifying one of the most abundant mRNAs in liver. Our objective was ambitious: we set out to reconstitute liver-specific albumin transcription in vitro using nuclear extracts from rat tissues. In vitro transcription had been accomplished with HeLa cell extracts in several laboratories, including those of the transcription wizards Bob Roeder and Bob Tjian. After many futile attempts, we finally succeeded in establishing cell-free transcription with nuclear extracts from rat tissues. These studies were greatly facilitated by Bob Roeder, who provided G-free cassette vectors for our experiments. To our delight, liver nuclear extracts generated about 50 times more RNA initiated at the albumin promoter than brain or spleen extracts, whereas all extracts yielded similar quantities of RNA initiated at the adenovirus major late promoter. These simple biochemical assays allowed us to delineate six albumin promoter elements, A to F, of which B and D were essential for efficient liver-specific in vitro transcription. The element B-binding factor was identified as HNF1 by Riccardo Cortese, Moshe Yaniv, and Jerry Crabtree, and major element D-binding proteins were shown to be C/EBP family members by my close friend Steve McKnight. So what was left for us? Chris Mueller, a Canadian postdoc in the lab, set out to identify additional site D-binding proteins by screening a bacteriophage cDNA expression library with radioactive oligonucleotides encompassing albumin promoter element D. Fortunately, he did find a novel D-box-binding transcription factor and dubbed it DBP (for albumin site D-Binding Protein). His discovery, published in *Cell*, secured him an assistant professorship at Queens College in Kingston, Canada, and me an extension of my Swiss National Science Foundation grant.

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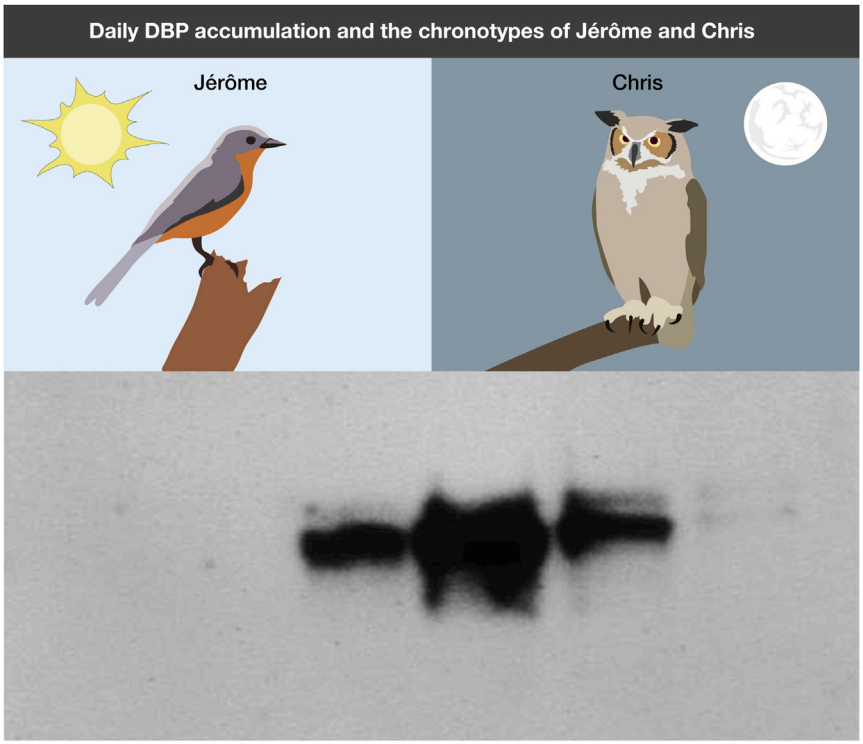
But what came next provoked a lot of anguish, frustration, and sleepless nights. After Chris left the lab, Jérôme Wuarin pursued work on DBP in the framework of his doctoral thesis. A few weeks later, he alerted me that DBP was a phantom. Using Chris' cDNA clones and antibodies as probes in northern and western blots, respectively, he repeatedly failed getting even traces of signals. This bad news was humiliating in two ways: it compromised my scientific reputation and challenged my knowledge of human nature. Chris always behaved as a generous, honest, and loyal lab citizen. It just seemed inconceivable to me that he fabricated scientific data, but I thought to myself, "we're going to have to retract the paper." Before composing a retraction letter for the *Cell* paper, I prepared liver nuclear extracts myself and handed them over to Jérôme for the "acid test." To our huge relief, a protein migrating with the expected molecular weight showed up in his western blot with my extracts, while his own samples looked like negative controls. How was this possible? After some discussion, it dawned on us that the time of day for sacrificing the rats were different in the experiments he, Chris, and I conducted. As the son of a farmer, Jérôme grew up waking early to tend to the cows. In the lab, he started work before 8 a.m. In contrast, Chris arrived in the lab late in the morning, and by the time he was ready to prepare his extracts, it was after 2 p.m. Since I was always busy with paperwork before lunch, my extracts also stemmed from rats euthanized during the afternoon. Jérôme's follow-up experiments were obvious. He prepared liver nuclear extracts at 4 hr intervals around the clock and examined them for the presence of



DBP by immunoblotting. The results were exhilarating. DBP accumulation oscillated by more than 100-fold during the 24 hr day, being undetectable during morning hours. Subsequent experiments revealed that transcriptional mechanisms accounted for circadian fluctuations in DBP expression. Owing to genome-wide transcriptomics studies in several labs, we now know that hundreds of genes are rhythmically transcribed in liver and other tissues. Ironically, however, *Dbp* is still on top of the list with regard to amplitude. We got lucky not by carefully planning experiments, but by pure chance, as Jérôme was a morning lark and Chris a night owl.

Soon after our discovery of DBP, the laboratories of Geoff Rosenfeld, Michael Clearly, and Thomas Look identified TEF and HLF, which, like DBP, possess a proline and acidic amino-acid-rich (PAR) peptide region and a basic leucine zipper (bZip) domain. Geoff and Thomas graciously provided *Tef* and *Hlf* reagents for our studies, and using them, we could demonstrate that TEF and HLF also follow a circadian expression cycle. Initially, our genetic loss-of-function experiments with PAR bZip genes were rather disappointing, as single- and double-knockout mice displayed only moderate phenotypes. Triple-knockout mice, however, were very frail. About half of the animals succumbed to a sudden death during the first three months of age, and the remaining half displayed an accelerated aging and early death phenotype. A short life expectancy is not surprising for progeria-affected animals. However, young triple-knockout mice looked perfectly vigorous, and we thus could not explain their sudden death. One day, Fred Gachon, a postdoc working on these animals, showed me a histogram on which he plotted death rate against weekdays. Stunningly, many more mice died on Mondays and Thursdays than on other weekdays. Did he discover a new clock ticking twice a week, whose activity was unmasked in PAR bZip null mice? We tentatively discarded this wild hypothesis because it violated Occam's razor. The answer emerged from conversations with the animal keepers: they cleaned the rooms hosting our knockout mice on Mondays and Thursdays, and it turned out that the sound of the vacuum cleaner provoked epileptic seizures in triple-knockout mice. The young mice dying on days other than Mondays and Thursdays also succumbed to spontaneous seizures, but these were less frequently fatal than the audiogenic attacks. So DBP and its cousins were good for a second surprise.

Dbp, *Tef*, and *Hlf* are clock output rather than core clock genes, since mice with null alleles for all three genes still displayed perfectly rhythmic locomotor activity cycles. *Clock* (for Circadian

locomotor output cycles kaput), the first mammalian core clock gene, was identified in 1997 by Joe Takahashi and his colleagues in a heroic forward genetics approach. Soon thereafter, mammalian homologs of the fruit fly gene *period* (*per*) were discovered through comparative genomics and PCR assays with degenerate primers in the laboratories of Chen Chi Lee and Hajime Tei, respectively. Two of the three mammalian period isoforms, *mPer1* and *mPer2*, turned out to have essential clock functions. Like PAR bZip genes, *mPer1* and *mPer2* were also found to be rhythmically expressed in peripheral tissues. Did this prove that all cells possess circadian clockworks? Not really; the daily oscillations in *mPer* gene expression could have been driven by rhythmic systemic signals orchestrated by the SCN master clock.

Aurélio Balsalobre, who performed his PhD thesis work on the impact of c-Fos on cell cycle progression and transformation of cultured Rat-1 fibroblasts, joined my lab as a postdoc in 1997, the year of mammalian clock gene discovery. The transcription of *c-Fos*, like that of many other immediate early genes, is rapidly induced by serum. Intriguingly, the expression of these genes is also stimulated by light pulses in the SCN, as demonstrated by work in Joe Takahashi's laboratory. Moreover, such light pulses reset the phase in circadian behavior. In 1997, Hitoshi Okamura and his colleagues observed that immediate early genes induced by light in the SCN include *mPer1* and *mPer2*. This discovery offered a plausible hypothesis for how the phase of the circadian master pacemaker can be reset by light. Encouraged by the findings of Joe and Hitoshi, Aurelio examined whether a serum shock could substitute for light in photo-insensitive Rat-1 fibroblasts in triggering immediate early *Per1* and *Per2* gene expression. The answer was unambiguously "yes." As RAT-1 cells express all known clock genes, the possibility existed, therefore, that these fibroblasts harbor hidden circadian oscillators. If every cell had a clock ticking in a different phase, circadian oscillations could obviously not be detected in populations containing millions of cells. Aurélio thus prepared a large number of dishes with confluent Rat-1 cells, added high concentrations of serum to induce a burst of PER1 and PER2 accumulation, prepared RNA samples at 4 hr intervals during three consecutive days, and examined the temporal accumulation of clock-related transcripts. On a late Saturday afternoon, he called me with a trembling voice, reporting that Rat-1 fibroblasts possess circadian clocks. To the dismay of my family, I left home at once and drove to the lab. When I saw his data, I immediately knew that they provided conclusive evidence for the existence of peripheral circadian clocks. A few months later, I called Ben Lewin, the founder and then editor-in-chief of *Cell*, asking him whether he was potentially interested in our story. "Send us the piece," was his succinct reply. Three weeks after submission, I received his editorial decision together with the reports of three reviewers. Ben wrote something like, "Dear Ueli, I am happy to say that all three reviewers are enthusiastic about your paper, and I am therefore glad to publish it in *Cell*, subject to minor revisions." What a shock when I read the comments of referees one and two. Both rejected the paper based on arguments like: "most unicellular organisms, even some bacteria, possess circadian clocks—so what's new?" The third referee was indeed enthusiastic. It must have been my friend Michael Rosbash, a foremost pioneer in circadian biology. In the *Cell* issue featuring our study (June 12, 1998), he wrote a flattering Preview: "Why the Rat-1 Fibroblast Should Replace the SCN as the In Vitro Model of Choice." In reaching editorial decisions, Ben Lewin clearly placed his judgment above that of the referees, albeit not always in favor of the authors. One could have very positive reviews, and the manuscript got nevertheless rejected because of "lack of enthusiasm by the referees." Nonetheless, *Cell*'s glory grew to a large part on Ben's good intuition and foresight.

When we published our paper on fibroblast clocks, we still did not know with certainty whether serum synchronized self-sustained yet out-of-phase oscillators or whether it triggered de novo oscillations of damped timekeepers. It took an additional six years until Emi Nagoshi, a postdoc in the lab, conclusively demonstrated the existence of self-sustained, cell-autonomous clocks in individual fibroblasts. In 2000, the research teams of Hajime Tei, Michael Menaker, and Joe Takahashi demonstrated that circadian clocks also kept ticking in various organ explants, thereby affirming the ubiquitous presence of peripheral oscillators. Our knowledge on how exactly these countless cellular clocks are synchronized in the body is still hazy, but feeding-fasting cycles, glucocorticoid signaling, actin polymerization cycles, and, somewhat surprisingly, body temperature rhythms appear to play important roles in this endeavor.

Richard Feynman, who was awarded the 1965 Nobel Prize in Physics for quantum electrodynamics (together with Sin-Itiro Tomonaga and Julian Schwinger), spent a one-year sabbatical in

the laboratory of Max Delbrück. Delbrück, a physicist converted to biologist, received the 1969 Nobel Prize in Physiology or Medicine for his pioneering work on bacteriophage genetics (together with Alfred Hershey and Salvador Luria). How did Feynman compare biology to physics? In his delightful biography, *Surely You're Joking, Mr. Feynman!*, he wrote, "So right away I found out something about biology: it was very easy to find a question that was very interesting, and that nobody knew the answer to. In physics you had to go a little deeper before you could find an interesting question that people didn't know." I am tempted to add: "In biology, it is easy to get interesting answers to questions one didn't ask."